



TITLE:

HISTOCHEMICAL STUDIES ON THE LIPID
REQUIREMENT FOR THE OXIDATIVE ENZYME
ACTIVITIES : I. STUDIES ON THE EFFECTS OF
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OXIDATIVE ENZYME ACTIVITIES

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CITATION:

OHKAWA, Kinichi. HISTOCHEMICAL STUDIES ON THE LIPID REQUIREMENT FOR THE OXIDATIVE ENZYME ACTIVITIES : I. STUDIES ON THE EFFECTS OF HISTOLOGICAL PROCEDURES FOR PREPARING TISSUE SECTIONS UPON THE OXIDATIVE ENZYME ACTIVITIES. *Acta tuberculosea Japonica* 1965, 15(1): 1-21

ISSUE DATE:

1965-09

URL:

<http://hdl.handle.net/2433/51701>

RIGHT:

HISTOCHEMICAL STUDIES ON THE LIPID REQUIREMENT FOR THE OXIDATIVE ENZYME ACTIVITIES

I. STUDIES ON THE EFFECTS OF HISTOLOGICAL PROCEDURES FOR PREPARING TISSUE SECTIONS UPON THE OXIDATIVE ENZYME ACTIVITIES

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(Received for Publication June 20, 1965)

INTRODUCTION

As is well known, the principle of enzyme histochemistry,^{1),2),3),4),5)} which has been widely applied in various branches of research in biology and medicine, is based on the catalytic activities of enzymes, which may be taken as one of the important biological attributes of enzyme protein. In practice, the principle generally consists of two processes; first, precipitation of a suitable end-product produced from a properly selected substrate by enzyme activity at the sites of enzyme localization; and second, microscopic visualization of precipitates of a suitable end-product by the formation of dye-stuff. Dye-stuff thus formed in the tissue sections does not combine directly with the molecular structure of enzyme protein. Therefore, the process of preparation of tissue sections is very important for the histochemical demonstration of enzymes, and it is reasonable that enzyme proteins should be well preserved in tissue sections throughout the whole process, and at the same time the catalytic activities of the enzymes also should be maintained in good condition. For the above-mentioned purposes it is desirable to preserve in good condition chemical materials which may play important roles in catalytic reactions of enzymes in the living mammalian body.

Fresh unfrozen tissue sections have been used generally for the histochemical demonstration of oxidases^{6),7),8)} and dehydrogenases^{9),10),11),12),13)} such as cytochrome oxidase, DPNH diaphorase, succinic dehydrogenase, malic dehydrogenase and so on, because their catalytic activities are lost or greatly diminished by fixation and embedding, histological procedures which are necessary for preparing paraffin sections. Moreover, it has been recognized

also that the catalytic activities of oxidative enzymes are almost lost or greatly reduced by dewaxing tissue sections of 5~6 μ thickness even when the activities withstand the procedures of fixation in chilled absolute acetone and embedding in paraffin with a melting point of about 48~50°C. This phenomenon is one of the interesting problems in histochemistry of oxidative enzymes.^{14),15),16),17),18),19)}

Particularly, cytochrome oxidase^{1),2),3),4)} has been designated synonymously "labile nadi reaction", and it has been claimed as an indispensable factor that for the histochemical demonstration of cytochrome oxidase activity the tissue sections must be fresh and unfixed. For the cytochrome oxidase activity is lost by the ordinary histological procedures.

It is the intention of the author to throw light on the causative factors in the inhibitory actions of these histological techniques such as fixation, embedding and dewaxing on the activities of oxidative enzymes. A careful study of the effects of chilled absolute acetone fixation, embedding in paraffin (melting point 48~50°C) and dewaxing, respectively, was performed. Paraffin with a melting point of 48~50°C will be designated as "soft paraffin" in the following description.

MATERIALS AND METHODS

Animal tissues used in the present experiments were obtained from various organs of normal adult male albino rats of Wistar strain weighing from 140 to 160 g. Small tissue blocks of each of the organs obtained immediately after sacrificing by decapitation, were dehydrated and fixed in several changes of chilled absolute acetone in a refrigerator (temperature about 3°C) during the initial 3 to 4 hours. Dehydration and fixation in chilled absolute acetone were continued for a total of 10~15 hours. The tissue blocks were then cleared in xylene for 30~45 minutes and embedded in soft paraffin.

Enzymes investigated in the present experiments with histochemical methods were lactic dehydrogenase and DPNH diaphorase. The composition of the substrate mixtures for the histochemical observation of each of these two enzymes was as follows:

(1) DPNH diaphorase

0.1% Nitro-blue tetrazolium	0.3 ml.
Sørensen's M/15 phosphate buffer (pH 7.4)	0.5 ml.
DPNH	5~10 mg.
Distilled water	0.2 ml.

(2) Lactic dehydrogenase

0.3 M Sod. lactate 0.4 ml.

0.1% Nitro-blue tetragolium 0.3 ml.

Sörensen's M/15 phosphate buffer (pH 7.4) 0.5 ml.

DPN 5~10 mg.

The pH of 0.3 M sod. lactate solution was adjusted to 7.0 with sod. hydroxide.

The histochemical method applied for bound lipids was the modified Berenbaum's method. The procedure was as follows:

- (1) Prepare tissue sections fixed in chilled absolute acetone and embedded in soft paraffin.
- (2) Stain in 1% Sudan Black B in absolute acetone at 37°C for 2~3 hours.
- (3) Wash in 80% alcohol containing acetone at the concentration of 1~3% for 5 minutes.
- (4) Mount in glycerine or gum syrup.

Experiment 1.

The experiment was done to compare the procedures of fixation of small tissue blocks in chilled absolute acetone and of embedding in soft paraffin with those of dewaxing tissue sections of 5~7 μ thickness, and to examine the changes in the activities of the two enzymes in each case.

Eight tissue sections of 6~7 μ thickness were prepared from the chilled absolute acetone fixed, soft paraffin embedded tissue blocks. These tissue sections were divided into two groups and each of them was used for the histochemical investigation of the activities of the two enzymes. For each of the two enzymes, four tissue sections were subdivided into two subgroups of two tissue sections; (a) and (b). Two tissue sections of group (a) were incubated in the substrate mixture of each of the two enzymes at 37°C without dewaxing. However, two tissue sections of group (b) were incubated in the substrate mixture of each of the two enzymes at 37°C after dewaxing with xylene, which was removed by graded alcohol series, and washing in water. Total incubation time ranged from 30 to 60 minutes.

Experiment 2.

This experiment was carried out to observe the effects upon the enzyme activities of thickness of the tissue sections used for incubation and of the organic solvents applied in the process of dewaxing.

- (1) Eight tissue sections of 30 μ thickness were prepared from the tissue blocks fixed in chilled absolute acetone and embedded in soft paraffin. Four

tissue sections each were used for the histochemical observation of DPNH diaphorase and lactic dehydrogenase activities, respectively. These four tissue sections were subdivided into two subgroups, (a) and (b). Two tissue sections of group (a) were incubated in the substrate mixture at 37°C after dewaxing with xylene, which was removed by graded alcohol series, and washing in water. However, tissue sections of group (b) were incubated in the substrate mixture after dewaxing with xylene, which was removed by absolute acetone, and washing in water. The total incubation time for the tissue sections of groups (a) and (b) were 30~60 minutes respectively.

(2) Eight tissue sections with 6~7 μ thickness were obtained from the same tissue blocks as in (1). Four tissue sections each were used for the histochemical observation of the activities of the two enzymes. These four tissue sections were divided into two subgroups, (a) and (b). Sections of group (a) were incubated in the substrate mixture at 37°C after dewaxing with xylene, which was removed by graded alcohol series, and washing in water. However, the tissue sections of group (b) were incubated in the substrate mixture at 37°C after dewaxing with xylene, which was removed by absolute acetone, and washing in water. Incubation time was 30~50 minutes.

Experiment 3.

This experiment was done to determine the effects on the activities of the enzymes when tissue sections were pre-treated with absolute acetone or with 90% aqueous acetone, respectively.

Eight tissue sections of 30 μ thickness were cut from the tissue blocks prepared in the same way as in the above experiments. Four tissue sections each were used for the histochemical demonstration of DPNH diaphorase and lactic dehydrogenase, respectively. The four tissue sections for each enzyme were further divided into two subgroups, (a) and (b). Two tissue section of group (a) before incubation were pre-treated with absolute acetone for 1 hour and 30 minutes after dewaxing with xylene, which was removed by absolute acetone, and washing in water. On the other hand, tissue sections of group (b) before incubation were pre-treated with 90% aqueous acetone for 1 hour and 30 minutes after dewaxing in the same way as that of group (a). The tissue sections of both groups were incubated in the substrate mixtures at 37°C for 30~60 minutes after pre-treatment.

Experiment 4.

This experiment was done to know to what extent lipids in tissue sections were extracted by the action of organic solvents in each procedure of histological techniques.

(1) Tissue sections of $6\sim7\mu$ thickness, which were cut from the chilled absolute acetone fixed, soft paraffin embedded tissue blocks, were stained with the modified Acetone-Sudan Black method without dewaxing.

(2) Tissue sections of $6\sim7\mu$ thickness, which were cut from the same tissue blocks as in (1), were stained with the modified Acetone-Sudan Black method after dewaxing with xylene, which was removed by absolute acetone.

(3) Tissue sections of $6\sim7\mu$ thickness were stained with the modified Acetone-Sudan Black method after dewaxing with xylene, which was removed by graded alcohol series.

(4) Tissue sections of 30μ thickness, which were cut from the chilled absolute acetone fixed, soft paraffin embedded tissue blocks, were stained with the modified Acetone-Sudan Black method after dewaxing with xylene, which was removed by absolute acetone.

(5) Tissue sections of 30μ thickness, which were cut from the same tissue blocks as in (4), were stained with the modified Acetone-Sudan Black method after dewaxing with xylene, which was removed by absolute acetone. In this case the tissue sections before staining were pre-treated with absolute acetone for 1 hour and 30 minutes.

(6) Tissue sections of 30μ thickness were stained with the modified Acetone-Sudan Black method after dewaxing with xylene, which was removed by absolute acetone. In this case the tissue sections before staining were pre-treated with 90% aqueous acetone for 1 hour and 30 minutes.

A comparative study of the changes in the enzyme activities observed in each of the experiments (1), (2) and (3) was made in relation to the results of (4).

RESULTS

(1) On comparing the enzyme activities both of group (a) and group (b) in experiment 1 the enzyme reactions of lactic dehydrogenase and DPNH diaphorase were recognized positively in the tissue sections incubated in the substrate mixture without dewaxing, and these positive reactions were strong enough for microscopic observation. In the tissue sections dewaxed with xylene, however, the activities of both enzymes were greatly reduced or had almost disappeared, and it was almost impossible to examine the enzyme activities histochemically under the microscope. That is to say, in the tissue sections of group (a) incubated before dewaxing there were recognized fine granular deposition of formazan dye in the cytoplasm of hepatic cells, epithelial cells of renal tubules and chief cells of gastric mucous membrane and

so on. The formation of formazan dye clearly indicates the occurrence of enzymatic reactions. These histochemical findings are shown in the pictures. These positive reactions were recognized to be due to specific enzyme activity, because in the control tests in which the substrates were removed, the positive reactions could not be recognized anywhere. There were no positive reactions recognized in any nuclei.

(2) The results obtained in experiment 2 were as follows :

(a) In the 30μ thick tissue sections of group (a) incubated in the substrate mixtures after dewaxing with xylene, which was removed by graded alcohol series and washing in water, the activities both of lactic dehydrogenase and DPNH diaphorase was greatly diminished or almost absent. In the tissue sections, however, of group (b) incubated after dewaxing with xylene, which was removed by absolute acetone and washing in water, the activities of both enzymes were strongly positive. Namely, to compare the results obtained from the experiments using tissue sections of 30μ thickness to which graded alcohol series was applied for removing xylene in the process of deparaffinization, with those of the experiments using tissue sections of 30μ thickness to which absolute acetone was applied for removing xylene, there could be recognized a distinct difference in the enzyme activities between the group (a) and group (b). The enzyme activities of group (a) were greatly reduced, while those of group (b) were almost unchanged.

(b) When the tissue sections of group (a) of $6\sim7\mu$ thickness were processed with graded alcohol series for removing xylene in deparaffinization, the enzyme activities of both lactic dehydrogenase and DPNH diaphorase were diminished almost to zero, while the enzyme activities in the tissue sections of group (b) processed with absolute acetone were often recognized positively in spite of great reduction. The aforementioned results indicate distinctly that the enzyme activity in tissue sections processed with graded alcohol series in dewaxing was almost nil, while that in tissue sections processed with absolute acetone in dewaxing was considerably reduced, though not to the same degree as the former.

(3) In experiment 3, to examine the histochemical enzyme activity in tissue sections of 30μ thickness which were pre-treated with absolute acetone and with 90% aqueous acetone respectively for about 1 hour and 30 minutes, the enzyme activity in tissue sections of group (a), pre-treated with absolute acetone, was a little reduced. However, that in tissue sections of group (b) pre-treated with 90% aqueous acetone was greatly diminished and only faint positive reactions were recognized microscopically. In short, there was a

distinct difference in the effect on the enzyme activity between the absolute acetone and the 90% aqueous acetone pre-treatment.

(4) There was a general tendency in the changes of bound lipid staining intensity that graded alcohol series and 90% aqueous acetone weakened the staining reaction. This may be assumed to be due to extraction of a considerable amount of bound lipids.

When the tissue sections of 6~7 μ thickness were not dewaxed the intensity of the staining with the modified Acetone-Sudan Black method was strong. This result indicates that bound lipids were not almost extracted from tissue blocks by fixation and embedding.

In the tissue sections of 6~7 μ thickness to which absolute acetone was applied for removing xylene in the process of dewaxing the intensity of the staining with the modified Acetone-Sudan Black method was moderate. This shows a little extraction of bound lipids from tissue sections. The weakness of the staining indicates that bound lipids such as phospholipids were extracted by absolute acetone to some extent.

When graded alcohol series was used for removing xylene from the tissue sections of 6~7 μ thickness the intensity of the staining reaction was very weak. This result indicates that a considerable amount of bound lipids were extracted by the action of alcohol.

When the tissue sections of 30 μ thickness were stained with the modified Acetone-Sudan Black method after dewaxing with xylene, which was removed by absolute acetone, the intensity of the staining reaction was still moderate. This result indicates that a considerable amount of bound lipids are still preserved in the tissue sections.

In the tissue sections which were pre-treated with absolute acetone the intensity of the staining reaction was weak. This indicates that bound lipids such as phospholipids were considerably extracted from the tissue sections by the pre-treatment with absolute acetone.

When the pre-treatment with 90% aqueous acetone was performed the intensity of the staining reaction with the modified Acetone-Sudan Black method was very weak. This indicates that most of bound lipids were extracted by 90% aqueous acetone.

Table 1 represents the summary of the relation between enzyme activity and bound lipids.

Bound lipids which were stained with the modified Acetone-Sudan Black method were reasonably assumed to be phospholipids. Because, according to Keilig chilled absolute acetone is able to extract glycerides, cholesterol,

Table 1.

<div>Various pretreatments of tissue sections</div> <div>Histochemical findings</div>	Tissue section of 6~7 μ thickness, without dewaxing	Tissue section of 6~7 μ thickness dewaxed with xylene. Xylene was removed by absolute acetone	Tissue section of 6~7 μ thickness dewaxed with xylene. Xylene was removed by graded alcohol series	Tissue section of 30 μ thickness dewaxed with xylene. Xylene was removed by absolute acetone	Tissue section of 30 μ thickness dewaxed with xylene. Xylene was removed by absolute acetone. Pretreated with absolute acetone for 1.5 hour	Tissue section of 30 μ thickness dewaxed with xylene. Xylene was removed by absolute acetone. Pretreated with 90% aqueous acetone for 1.5 hour
Intensity of the staining reaction with the modified Sudan-Black B method for bound lipids	(\equiv)	($+$)	($+$)	(\equiv)	($+$)	($\pm \sim +$)
Intensity of the histochemical enzyme activities of DPNH diaphorase and lactic dehydrogenase	(\equiv)	($\pm \sim +$)	($- \sim \pm$)	($+$)	($\pm \sim +$)	($- \sim \pm$)

(\equiv) : Strongly positive ($+$) : Moderately positive ($+$) : Weakly positive
 (\pm) : Trace ($-$) : Negative

cholesterol esters and other steroids from tissue blocks, bounds lipids preserved in the tissue sections which were cut from the small tissue blocks fixed in chilled absolute acetone for 10~15 hours are assumed to be phospholipids. Therefore, table 1 indicates the outline of the relation between enzyme activity and phospholipids. It is able to obtain an evidence that phospholipids participate in the manifestation of catalytic activities of DPNH diaphorase and lactic dehydrogenase.

DISCUSSION

The intracellular conditions of existence of the target enzyme are of great importance in the histochemical demonstration of various enzymes. In relation to the techniques for preparing tissue sections it is important to know whether an enzyme to be demonstrated with histochemical methods is in strict combination with a particular intracellular fine structure or exists in a fluid state without any special morphological relation to the intracellular structures.¹⁹⁾ For the above-mentioned purposes data of biochemical studies often give us many useful suggestions. Enzymes in fluid state may be lost by flowing out of the cells during the process of preparing tissue sections or during incubation in the substrate mixture, or they may diffuse into the areas adjacent to the original sites of enzyme localization even though they may not flow out of the cells. On the other hand, in case of enzymes in strict combination with a certain intracellular fine structure it is relatively easy to

prepare good tissue sections for enzymorphological purposes, because the danger of diffusion of enzymes is much less than in the former. In the latter instance, however, participation of other factors related to the intracellular structures may complicate the situation. For instance, it has been reported recently with the histochemical methods that the activities of DPNH diaphorase, succinic dehydrogenase, malic dehydrogenase, β -hydroxybutyric dehydrogenase and sorbitol dehydrogenase are caused to disappear or to be greatly reduced by the histological procedures of fixation, embedding and dewaxing in spite of the fact that these enzymes are said biochemically to localize mainly in the mitochondrial membranes.^{19),20),21),22),23),24)} Moreover, even if the tissue sections prepared by freezing and drying are used for the histochemical demonstration of succinic dehydrogenase activity, it has been reported by M. S. Burstone that the activity of the enzyme is much reduced by the procedures of dewaxing.^{5),14)} At present it is not clearly known why the activities of these enzymes are reduced or lost in the preparation of tissue sections. The purpose of the present experiments is to clarify the causes of reduction of enzyme activity in such cases.

In experiment 1, since the tissue sections of group (a) were incubated in the substrate mixtures without dewaxing, histochemical findings in this group may be taken to indicate the effects of the procedures of fixation in chilled absolute acetone and embedding in soft paraffin upon the activities of lactic dehydrogenase and DPNH diaphorase. On the other hand, histochemical findings of group (b) indicate the effects of dewaxing upon the enzyme activities in addition to those of group (a). The following is a summary of the processes of the two groups:

Thickness of tissue section	Histological procedures (fixation, embedding and dewaxing) Numbers in parentheses indicate the duration of organic solvent action.	Enzyme activity
Group (a) 1.5~2.0 mm.	Absolute acetone \rightarrow Xylene \rightarrow (10~15 hours) (30~40 min.) \rightarrow Soft paraffin (30~45 min.)	(+)
Group (b) 6~7 μ	Xylene \rightarrow Absolute alcohol \rightarrow (2~3 min.) (1~2 min.) \rightarrow 80% Alcohol \rightarrow 60% Alcohol \rightarrow (1~2 min.) (1~2 min.) \rightarrow Washing in water (2~3 min.)	(-)

As is easily understood from the above-mentioned summary, the main differences between group (a) and group (b) are thickness of tissue sections, kind of organic solvents and time of action, and the enzyme activities. Of these main differences the two most important differences, which may seriously influence the histochemical enzymatic reactions, are the thickness of tissue sections and the kind of organic solvents. In comparing the enzyme activities of the two groups it may be reasonably assumed that heating at about 50°C for about 45 minutes in embedding tissue blocks in soft paraffin, and exposure to absolute acetone for about 10~15 hours in fixation and to xylene in clearing tissue blocks for about 45 minutes do not cause any serious inhibitory influence on the enzyme activity. Alcohol used in group (b) for removing paraffin from tissue sections may be regarded as an inhibitory reagent causing adverse effects upon the histochemical enzyme reaction. It is evident that the procedures of fixation in chilled absolute acetone and of embedding in soft paraffin are not inhibitory to the enzyme activity.

The purpose of experiment 2 was to make comparative studies of the above-mentioned two main differences. The outlines of the experiments are as follows:

- (1) Cases of tissue sections of 30 μ thickness (fixed in chilled absolute acetone and embedded in soft paraffin)

Processes of dewaxing.	Enzyme activity
Group (a) Dewaxing with xylene \rightarrow Absolute alcohol \rightarrow \rightarrow (80% Alcohol \rightarrow 60% Alcohol) \rightarrow \rightarrow Washing in water	(–)~(±)
Group (b) Dewaxing with xylene \rightarrow Absolute acetone \rightarrow \rightarrow Washing in water	(+)
- (2) Cases of tissue sections of 6~7 μ thickness (fixed in chilled absolute acetone and embedded in soft paraffin)

Processes of dewaxing	Enzyme activity
Group (a) Dewaxing with xylene \rightarrow Absolute alcohol \rightarrow (80% Alcohol \rightarrow 60% Alcohol) \rightarrow \rightarrow Washing in water	(–)
Group (b) Dewaxing with xylene \rightarrow Absolute acetone \rightarrow \rightarrow Washing in water	(–)~(±)

In comparing the above-mentioned processes it may reasonably be concluded that the essential factor which dominates the histochemical enzyme reaction is not the thickness of the tissue sections but the organic solvents

used in the dewaxing processes. Of the organic solvents used in dewaxing processes, alcohol has an inhibitory action on the enzyme activity. However, as the exposure to alcohol was brief, it can not be reasonably deduced that enzyme proteins had been denatured by alcohol treatment and that denaturation caused a great reduction in, or disappearance of the enzyme activity.²⁵⁾ It is more reasonable to assume that there are certain chemical substances in tissue cells performing important roles in normal enzyme activities, and that these chemical substances are soluble in alcohol but almost insoluble in absolute acetone. Therefore, reduction or disappearance of the enzyme activity may be due to the loss of these important chemical substances from the cells by extraction with the alcohol used in dewaxing processes.

Phospholipids provide an example of chemical substances which exhibit such typical properties in relation to organic solvents as mentioned above.^{1), 2), 4), 26), 27)} Phospholipids form one of the main structural components of the cells, with special relationship to mitochondria and other intracellular ultra-structure.^{27), 28), 29), 30), 31), 32), 33)} According to the unit membrane theory proposed by J. D. Robertson in 1957,³⁵⁾ phospholipid has been claimed to be one of the important components of the triple-layered unit membrane structure. Therefore, phospholipids are presumably required for the normal ultrastructural arrangement of some oxidative enzymes and the electron transfer among these enzymes. E. R. Redfearn reported in 1961²⁸⁾ that phospholipids amounted to 90.1% of the total lipid of pig heart muscle mitochondrial preparation.

Where absolute acetone is used for removing xylene in dewaxing processes

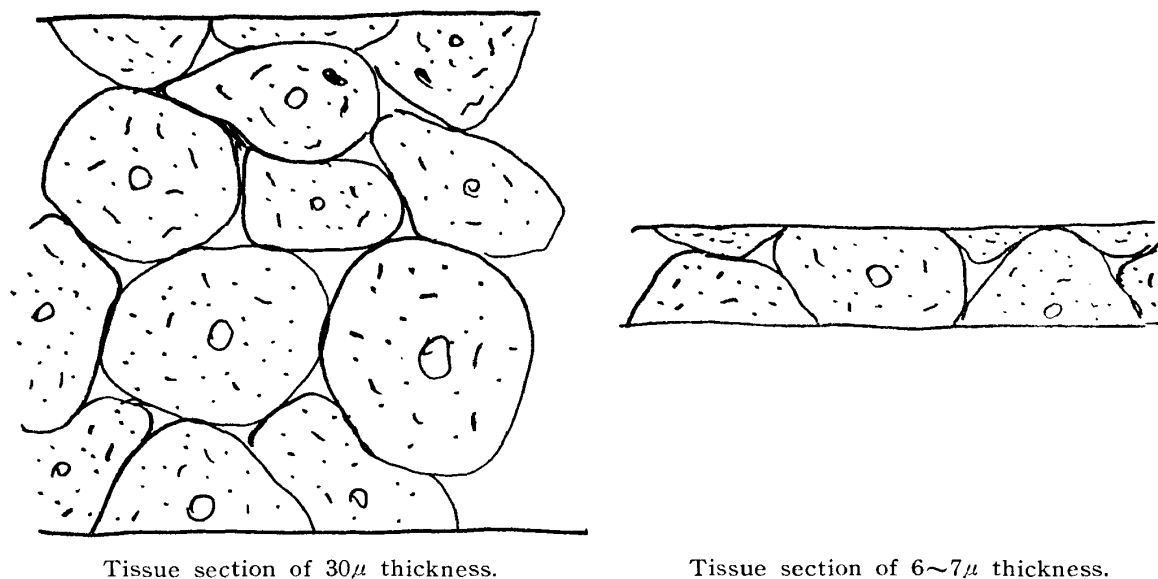


Fig. 1.

the relationship of enzyme activity and the thickness of the tissue sections is as follows: As shown in the pictures above, there are possibly some cells with intact cell membranes in 30μ thick tissue sections, while in sections of $6\sim7\mu$ thickness it is doubtful that cells with intact membranes remain after sectioning. Absolute acetone may not easily enter cells with intact cell membranes to extract phospholipids, but it can easily attack cells with damaged cell membranes. Therefore, it is reasonable to think that the greater reduction of enzyme activity in tissue sections of $6\sim7\mu$ thickness than that in sections of 30μ thickness is due to destruction of the cell membranes, rather than the thickness of sections *per se*. Although it has been generally accepted that absolute acetone does not dissolve phospholipids, according to the report of Shapiro *et al.*, cited in the book written by L. Lison,³⁾ cellular phospholipids that are in a mixed state with other lipids such as cholesterol esters and neutral lipids, can be extracted to some extent by absolute acetone. This is probably one reason why the enzyme activity decreases even on applying absolute acetone for removing xylene.

In biochemical studies on the electron transfer system of mitochondria S. Fleischer *et al.*^{34),36),37)} have reported that more than 80% of mitochondrial phospholipids were extracted with 90% aqueous acetone from isolated mitochondria, without any significant morphological changes, and that when mitochondria were treated with 90% aqueous acetone, thus losing almost all of their phospholipids, the enzyme activities of succinatecytochrome C reductase and cytochrome oxidase almost disappeared. Therefore, if in the present experiments the extraction of phospholipids actually resulted in the reduction or disappearance of the enzyme activity, it is natural to expect that the 90% aqueous acetone treatment will cause the reduction or disappearance of enzyme activity.

This possibility was studied in experiment 3, the outlines of which are described below:

	Enzyme activity
Group (a) Dewaxing with xylene → Rinse in absolute acetone → → Washing in water → Pre-treatment with absolute acetone for one hour and 30 min.	(+)
Group (b) Dewaxing with xylene → Rinse in absolute acetone → → Washing in water → Pre-treatment with 90% aqueous acetone for one hour and 30 min. (thickness of the tissue sections was 30μ .)	(-)(±)

When the tissue sections were pre-treated with absolute acetone for one hour

and 30 minutes the enzyme reactions were clearly positive, though the activity was reduced almost to half the normal degree. However, on pre-treatment with 90% aqueous acetone the activities of the enzymes was reduced to almost zero and formazan dye was rarely formed. It is reasonable to think that the disappearance of the enzyme activity was due to the loss of phospholipids from the cells by pre-treatment with 90% aqueous acetone. The enzyme activity of the tissue sections pre-treated with absolute acetone was considerably reduced, because absolute acetone also is able to dissolve cellular phospholipids that are in a mixed state with other lipids.

By the histochemical experiments described above, phospholipids have been proved to be substances of great importance for the activity of some oxidative enzymes.

In experiment 4, changes in the intensity of the staining reaction with the modified Acetone-Sudan Black method were in parallel with the changes in the enzyme activity. This result may also supports the author's opinion that phospholipids are important for the activities of some oxidative enzymes. That is, the extraction of cellular phospholipids results in the reduction or disappearance of the activity of some oxidative enzymes.

Although it has been generally stated that lactic dehydrogenase, DPNH diaphorase and some other oxidative enzymes lose their activities by fixation, embedding, and dewaxing the tissue sections fixed in chilled absolute acetone and embedded in soft paraffin, and also by dewaxing tissue sections prepared by freeze-drying and embedding in soft paraffin, it has been proved that the cause of the loss of the enzyme activity in these cases is the loss of cellular phospholipids by extraction with organic solvents such as alcohol.^{28),29),30),31),32),33),34),36),37),38),39),40),41),42)} Therefore, if phospholipids are preserved in tissue sections after histological procedures such as fixation, it will be able to demonstrate enzyme activity in tissue sections fixed in properly selected fixatives. This may allow to obtain much better histochemical findings than the usual methods using unfixed tissue sections.

SUMMARY

(1) In enzyme histochemistry the processes for preparation of tissue sections are very important, and it has been clearly proved that the reduction or disappearance of lactic dehydrogenase and DPNH diaphorase activities by fixation, embedding and dewaxing was caused by alcohol, one of the organic solvents used in the histological processes.

(2) The cause of the inhibitory action of alcohol has been demonstrated to lie in the extraction of phospholipids out of the cells.

(3) Although the mechanism of action of phospholipids is not evident at present, it has been made clear that phospholipids play an important role in manifestation of the activities of lactic dehydrogenase and DPNH diaphorase.

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EXPLANATIONS OF MICROPHOTOGRAPHS

(1) Kidney: 100×

DPNH diaphorase activity. Tissue section is 6μ thick. Tissue section which was fixed in absolute acetone and embedded in soft paraffin, was incubated in substrate mixture without dewaxing. The activity of the enzyme is localized in the cytoplasm of epithelial cells of urinary tubules. No positive reaction is recognized in any nuclei. In glomeruli are also seen no positive reaction.

(2) Kidney: 200×

DPNH diaphorase activity. Higher magnification of the previous picture. It is clearly shown in this picture that no positive reaction is recognized in any nuclei. The activity of the enzyme is very strong in the cytoplasm of the epithelial cells of urinary tubules.

(3) Intestine: 100×

DPNH diaphorase activity. Tissue section, which is 6μ thick, was prepared from tissue block, fixed in absolute acetone and embedded in soft paraffin, was incubated in substrate mixture without dewaxing. The intense activity of the enzyme is seen in the epithelial cells. Moderate activity is seen in the submucous tissue.

(4) Liver: 100×

Lactic dehydrogenase activity. Tissue section of 6μ thickness, which was prepared from tissue block fixed in absolute acetone and embedded in soft paraffin, was incubated in substrate mixture without dewaxing. It is clear that intense activity of the enzyme is localized in the cytoplasm of hepatic cells and that no positive reaction is recognized in any nuclei.

(5) Liver: 200×

Lactic dehydrogenase activity. Higher magnification of the previous picture. It is clearly recognized that positive reaction is not localized in nuclei.

(6) Kidney: 100×

Lactic dehydrogenase activity. Tissue section of 6μ thickness was incubated in substrate mixture without dewaxing. Strong positive reaction is recognized in the epithelial cells of proximal convolutions and loops of Henle. However, no positive reaction is clearly recognized in any glomeruli.

(7) Kidney: 200×

Lactic dehydrogenase activity. Higher magnification of the previous picture. In this picture it is easily recognized that no positive reaction is localized in any nuclei.

(8) Liver: 100×

Lactic dehydrogenase activity. Thickness of tissue section is 30μ . Tissue section was prepared from tissue block which was fixed in absolute acetone and embedded in soft paraffin. Tissue section, thus prepared, was incubated in substrate mixture after dewaxing. Xylene used in the dewaxing process was removed by absolute acetone. When alcohol was used for removing xylene, the activity of the enzyme was greatly reduced. It is clearly shown that the activity of the enzyme is localized in the cytoplasm of hepatic cells.

(9) Kidney: 100×

Lactic dehydrogenase activity. Tissue section of 30μ thickness was incubated in substrate mixture after dewaxing. Xylene was removed by absolute acetone. Intensely positive reaction is recognized in the epithelial cells of the urinary tubules.

(10) Liver: 100×

Lactic dehydrogenase activity. Thickness of tissue section is 30μ . Tissue section was treated with absolute acetone for 1 hour and 30 minutes before incubation. The activity of the enzyme was considerably reduced by the pre-treatment with absolute acetone. However, positive reaction is clearly recognized in the cytoplasm of hepatic cells.

(11) Stomach: 100×

Lactic dehydrogenase activity. Tissue section was treated with absolute acetone for 1 hour

and 30 minutes before incubation. The activity of the enzyme is considerably reduced by the pre-treatment with absolute acetone. However, positive reaction is seen.

(12) Stomach: 200×

Lactic dehydrogenase activity. Higher magnification of the previous picture. It is evident that no positive reaction is localized in any nuclei.

(13) Pancreas: 100×

Sudan Black B staining for bound lipid. Tissue section of 6 μ thickness was stained with Sudan Black B solution after dewaxing with xylene. Xylene was removed by absolute acetone. The intensity of the staining reaction is very strong in pancreatic cells.

(14) Kidney: 100×

Sudan Black B staining for bound. Tissue section of 6 μ thickness was stained with Sudan Black B solution after dewaxing with xylene which was then removed by absolute acetone. The intensity of the staining reaction is strong, especially in the basement membrane.

(15) Liver: 100×

Sudan Black B staining for bound lipid. Tissue section of 6 μ thickness was stained with Sudan Black B solution after dewaxing with xylens which was removed by absolute acetone. The intensity of the staining is strong.

(16) Pancreas: 100×

Sudan Black B staining for bound lipid. Tissue section of 6 μ thickness was stained with Sudan Black B solution after dewaxing with xylene which was removed by graded alcohol series. In this case the intensity of the staining is much reduced. It is evident that bound lipid was extracted to some extent by graded alcohol series.

(17) Intestine: 100×

Sudan Black B staining for bound lipid. Tissue section of 6 μ thickness was stained with Sudan Black B solution after dewaxing with xylene which was removed by graded alcohol series. The intensity of the staining is much reduced in some areas. The reduction of the intensity of the staining evidently indicates the extraction of a certain amount of bound lipid.

(18) Heart: 100×

Sudan Black B staining for bound lipid. Tissue section of 6 μ thickness was stained with Sudan Black B solution after dewaxing with xylene which was removed by graded alcohol series. The intensity of the staining reaction is considerably reduced.

(19) Skeletal muscle: 100×

Sudan Black B staining for bound lipid. Tissue section of 30 μ thickness was dewaxed with xylene which was removed by absolute acetone. The dewaxed tissue section was pre-treated with absolute acetone for 1 hour and 30 minutes before staining with Sudan Black B solution. The intensity of the staining was reduced to some extent. Reduction of the staining intensity evidently indicates that even absolute acetone is able to extract bound lipid.

(20) Heart: 100×

Sudan Black B staining for bound lipid. Tissue section was also pre-treated with absolute acetone for 1 hour and 30 minutes as in the case of (19). The staining intensity is reduced to some extent. Thickness of tissue section is also 30 μ .

(21) Stomach: 100×

Sudan Black B staining for bound lipid. Tissue section of 30 μ thickness was pre-treated with absolute acetone for 1 hour and 30 minutes before staining. The intensity of the staining reaction was reduced.

(22) Skeltal muscle: 100×

Sudan Black B staining for bound lipid. Tissue section of 30 μ thickness was pre-treated with 90% aqueous acetone for 1 hour and 30 minutes before staining. The intensity of the staining reaction is much reduced. The degree of reduction of the staining intensity is stronger than that of the pre-treatment with absolute acetone and it evidently shows that

the amount of bound lipid extracted by 90% aqueous acetone is larger than that of bound lipid extracted by absolute acetone.

(23) Heart: 100×

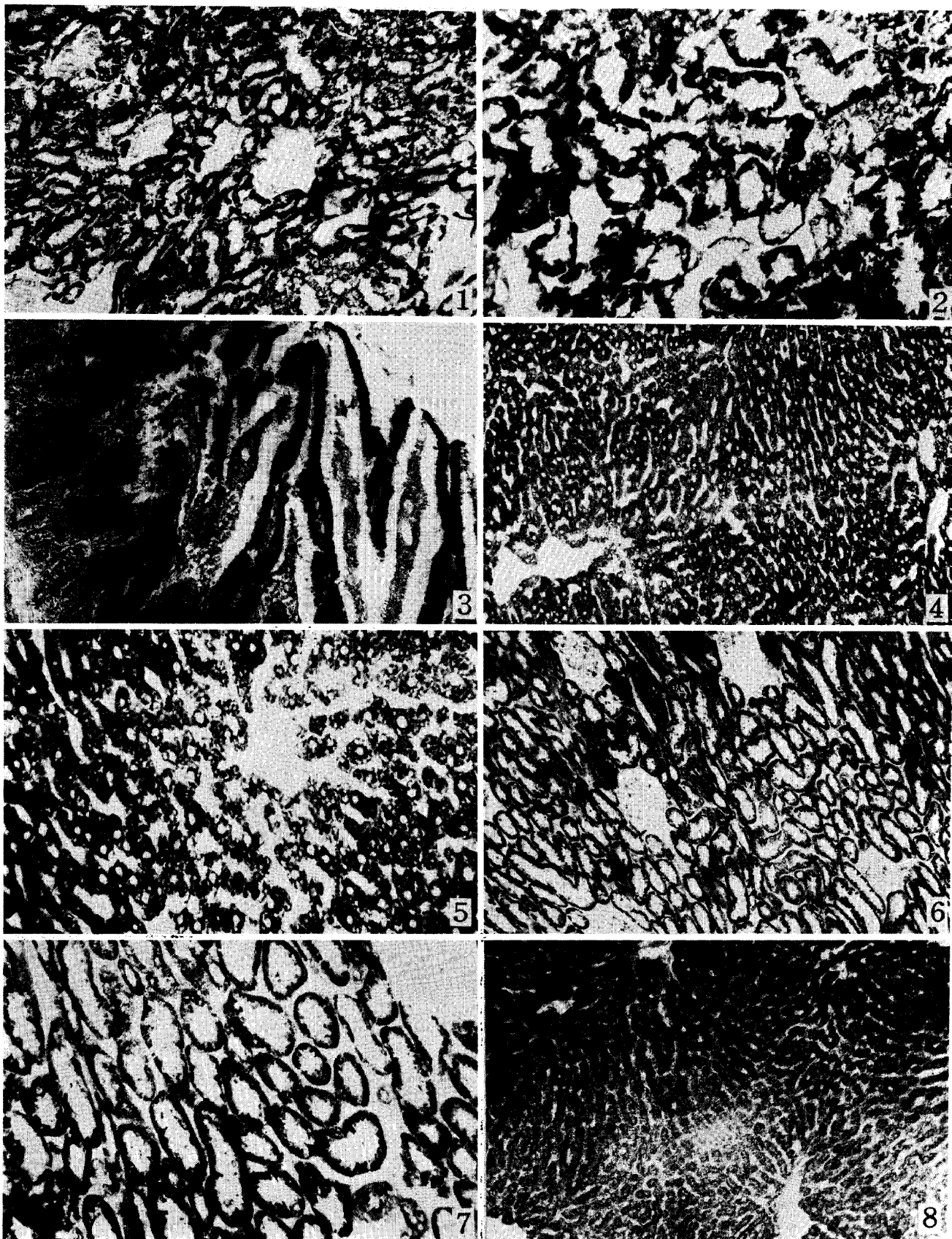
Sudan Black B staining for bound lipid. Thickness of tissue section was 30 μ . Tissue section was pre-treated with 90% aqueous acetone for 1 hour and 30 minutes. The intensity of the staining reaction is much reduced.

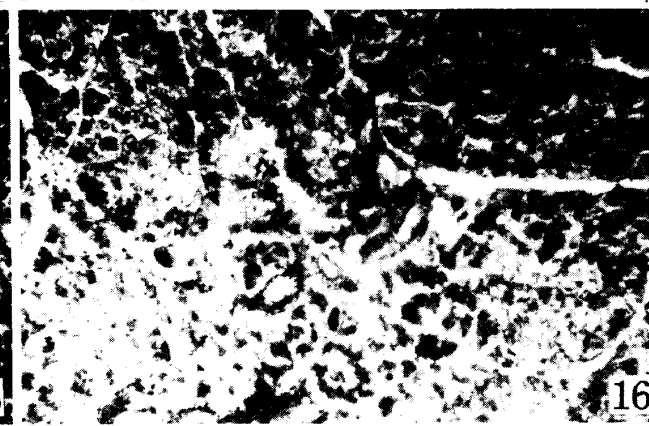
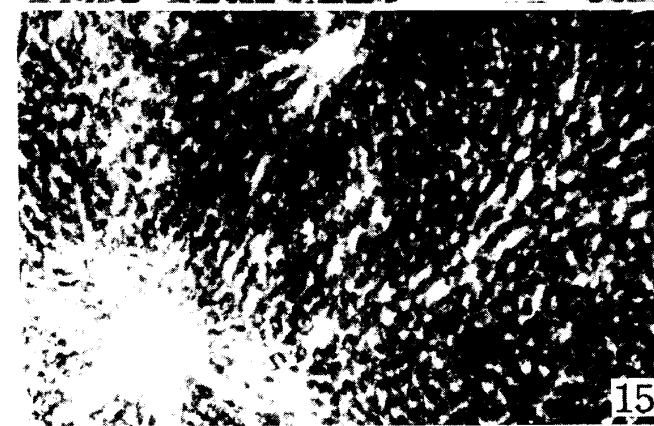
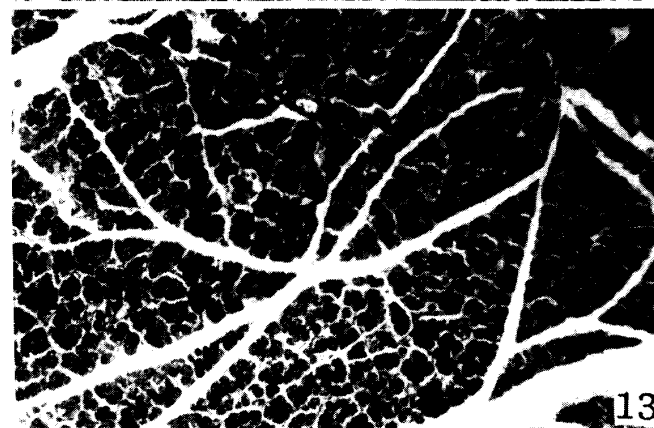
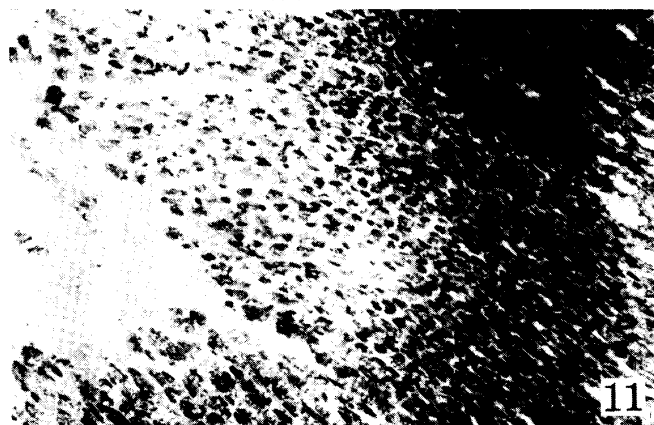
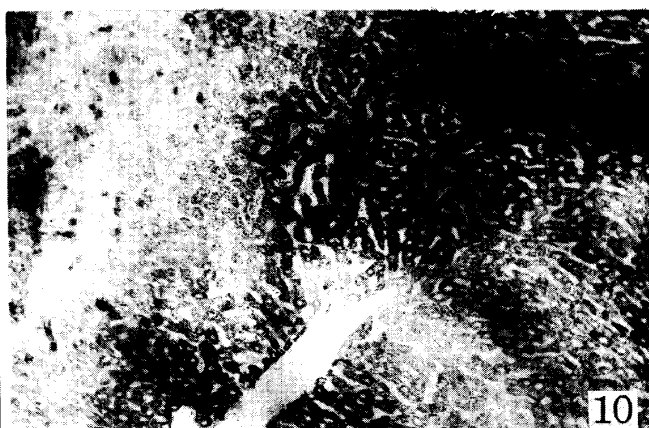
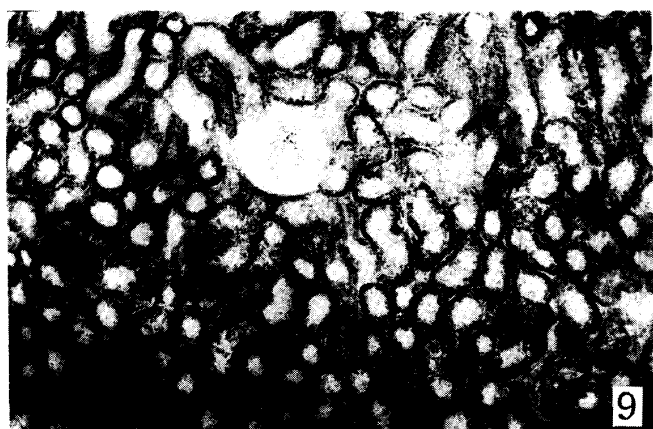
(24) Stomach: 100×

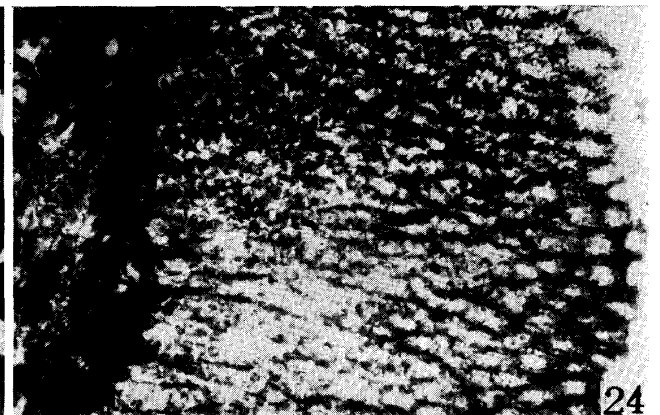
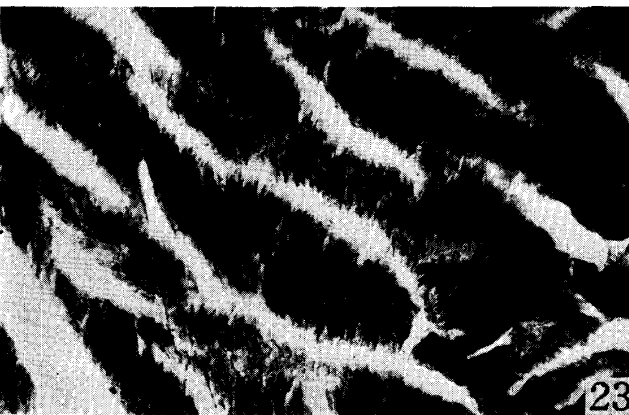
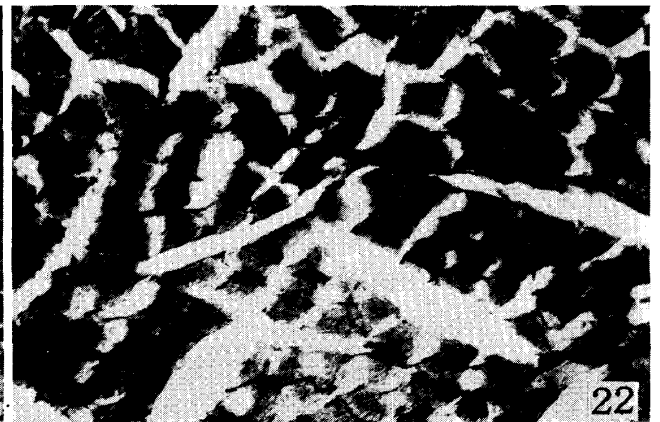
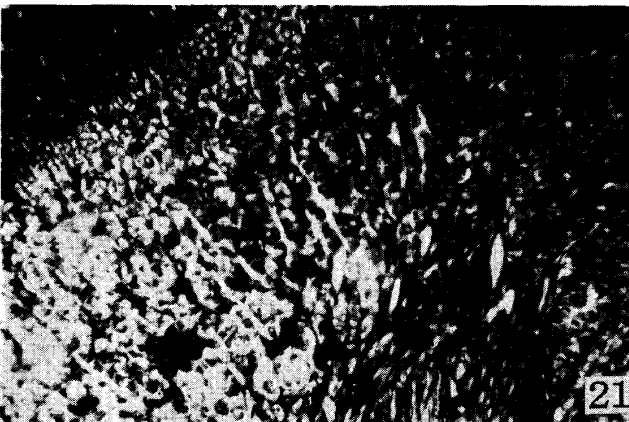
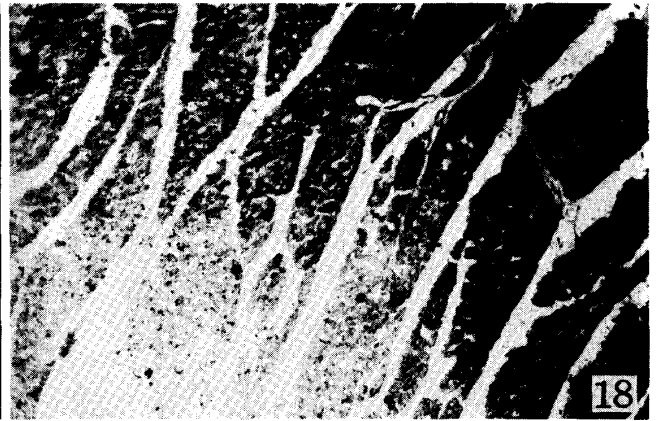
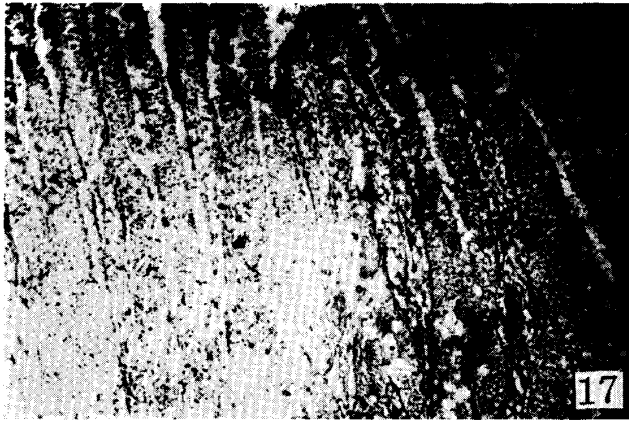
Sudan Black B staining for bound lipid. Tissue section is 30 μ thick. Tissue section was pre-treated with 90% aqueous acetone for 1 hour and 30 minutes. The intensity of the staining reaction is much reduced.

(25) A₁ and A₂ are lactic dehydrogenase activity. A₁ shows the activity of lactic dehydrogenase in tissue sections which were pre-treated for 1 hour and 30 minutes with absolute acetone. A₂ shows the activity of the enzyme in tissue sections which were pre-treated with 90% aqueous acetone for 1 hour and 30 minutes before incubation. The enzyme activity of A₁ is stronger than that of A₂. B₁ and B₂ show DPNH diaphorase activity. B₁ indicates the activity of DPNH diaphorase in tissue sections pre-treated with absolute acetone for 1 hour and 30 minutes. B₂ shows the activity of the enzyme in tissue sections pre-treated with 90% aqueous acetone for 1 hour and 30 minutes before incubation.

It is evident that the activity of the enzyme of B₁ is stronger than that of B₂

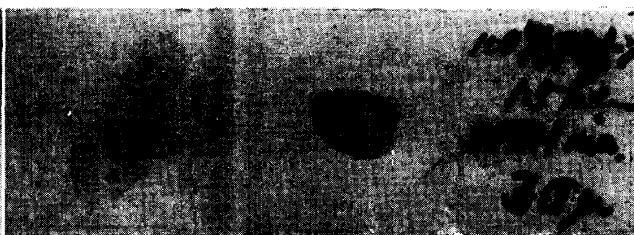




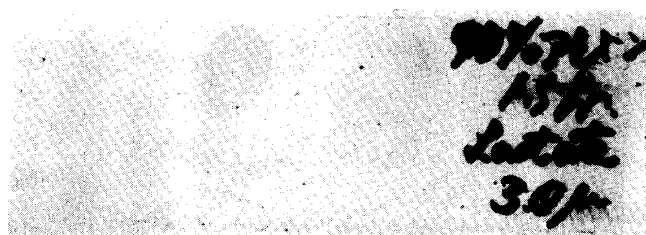




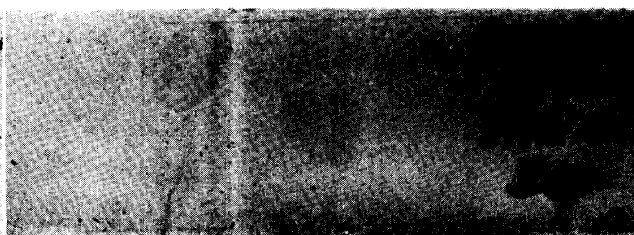
A₁



B₁



A₂



B₂